Provisional chapter

DNA Damage in End-Stage Renal Disease Patients. Assessment by *In Vitro* Comet Assay and by Cell-Free DNA Quantification

Susana Coimbra, Alice Santos-Silva, Elísio Costa and

Elsa Bronze-da-Rocha

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Abstract

Inflammation is a common feature in end stage renal disease (ESRD) patients on hemodialysis (HD) that might contribute to increase DNA damage. Inflammation biomarkers, such as C-reactive protein (CRP) and pro-inflammatory cytokines, are frequently raised in chronic kidney disease (CKD). It has been reported that patients with CKD present increased levels of circulating cell-free DNA (cfDNA) and different types of DNA injury. The genomic damage at the level of a single cell can be recognized by comet assay, a sensitive and simple technique to detect cell DNA alterations. The underlying inflammatory process in ESRD patients under HD therapy may be associated with increased genomic damage and cfDNA contributing to further enhance inflammation and the associated morbidity and mortality risk. In a recent work, we analyzed the degree of genomic damage in ESRD patients under HD therapy for more than 1 year, using the alkaline *in vitro* comet assay and the cfDNA quantification, to evaluate DNA damage within the cell and the circulating free DNA. We found that ESRD patients presented significantly higher CRP values and cell damaged DNA. The cfDNA values were correlated with age and inflammatory stage. Nine out 39 patients died during the 1 year follow-up period and presented significantly higher cfDNA, than those who persisted alive. Our data suggest that at lower CRP values, the increased DNA damage is still within the cell, and at higher CRP values the damaged DNA is released in to plasma. The higher degree of genomic damage in ESRD patients might be a consequence of inflammation and aging, and may contribute to increase the risk for cancer and cardiovascular (CV) mortality. Moreover, our data suggest that the comet assay is more sensitive for low grade inflammatory conditions, while cfDNA appears as a good biomarker for more severe inflammatory conditions, as well as a biomarker for the outcome of ESRD patients. In summary, the genomic damage in ESRD patients seems to result, at least in part, from inflammation and aging, and may contribute to increase the risk for cancer and CV mortality.

Keywords: chronic kidney disease, end-stage renal disease, inflammation, genomic damage, comet assay, cell-free DNA

1. Introduction

Kidneys are important in homeostasis, ensuring the excretion of toxic substances and regulating blood volume, blood pressure, concentration of electrolytes, plasma osmolarity and the acid/base balance. The kidneys also have endocrine functions, producing hormones, such as erythropoietin and calcitriol.

Chronic kidney disease (CKD) is characterized by a decline in kidney function and/or altered renal structure, leading to a gradual to permanent loss of kidney function over time. End-stage renal disease (ESRD), the worst stage of chronic kidney disease (CKD), requires dialysis to prevent accumulation of toxins, excessive water and electrolytes, or kidney transplantation [1].

Inflammation is a common feature in CKD, especially enhanced in ESRD patients on hemodialysis (HD). This chronic inflammatory state seems to contribute to aggravate kidney dysfunction and favor the occurrence of comorbidities and the risk of mortality [2, 3].

Chromosomal abnormalities, reduced DNA repair and DNA lesions have been reported in CKD patients [4]; increased levels of circulating cell-free DNA (cfDNA) [5], DNA-histone complexes [6] and different types of DNA injury [4, 7, 8] were also reported. The DNA-histone complexes have been proposed as markers of cardiovascular (CV) events in CKD and ESRD patients [6]. These genetic changes may explain, at least in part, the increased risk of cancer in these patients. In ESRD patients the HD treatment seems to contribute *per se* to enhance inflammation and, thus, it may also favor genetic damage and the associated complications [9, 10].

2. Chronic kidney disease and inflammation

CKD is associated with high mortality rates and its prevalence is increasing worldwide. The five clinical stages of CKD are based on the values of glomerular filtration rate and albuminuria (**Table 1**). At stages 1 and 2, patients are usually asymptomatic, presenting kidney damage and/or loss of kidney function. Stages 3 and 4 are associated with deterioration of renal function, from mild to severe dysfunction. In stage 5 (or ESRD), loss of kidney function is irreversible and the patients need renal replacement therapy [1, 11, 12]. Given the increasing prevalence of ESRD patients on HD treatment, CKD is a major health public problem, with significant socio-economic consequences and a considerable impact on functional status and quality of life of patients [13].

Diabetes *mellitus* and arterial hypertension are the two most common causes of CKD [15, 16]. Other possible causes, although less common, include glomerulonephritis, nephrolithiasis, pyelonephritis and polycystic kidney disease [17, 18].

		Albuminuria (mg/g)		
CKD stages	**GFR (ml/min/1.73 m²)**	**<30**	**30–300**	**>300**
1	≥90	LR	MIR	HR
2	60–89	LR	MIR	HR
3a	45–59	MIR	HR	VHR
3b	30–44	HR	VHR	VHR
4	15–29	VHR	VHR	VHR
5	<15	VHR	VHR	VHR

LR, low risk; MIR, moderately increased risk; HR, high risk; VHR, very high risk.

Table 1. Prognosis of chronic kidney disease (CKD), according to glomerular filtration rate (GFR) and albuminuria (adapted from Ref. [14]).

Regardless of technologic improvements in dialysis, ESRD is associated with substantial morbidity and mortality risks [19]. Actually, the improvements in dialysis procedures and in membrane flux, with higher clearance of small solutes, do not necessarily improve patient's survival [20, 21].

In CKD patients, the CV disease (CVD) events are the most frequent causes of death [22], while infections and malignancies are the most common non-cardiovascular causes, particularly in ESRD patients on HD. The high incidence of CVD in CKD patients has been associated with the high prevalence of traditional and non-traditional CV risk factors. Diabetes mellitus, arterial hypertension, dyslipidemia, obesity, sedentarism, smoking habits and age are important traditional CVD risk factors. Non-traditional CVD risk factors in CKD patients are more specifically related to the disease itself and/or to dialysis associated complications (e.g., inflammation, anemia, oxidative stress, hyperphosphatemia, left ventricular hypertrophy, endothelial dysfunction, insulin resistance and high levels of lipoprotein(a)). Comorbidities, such as infection, inflammation, oxidative stress, iron deficiency, anemia, vascular calcification, uremia and volume overload, are associated with a poor outcome and increased mortality risk in patients undergoing HD [3, 23–25]. Associations of these risk factors in CKD patients seem to represent a cumulative and additive risk for CV events. Actually, it has been difficult to find a biomarker or a panel of biomarkers that allows the evaluation/prognostic of the clinical condition. This is particularly complex for ESRD patients in HD, as they present several processes associated with renal tissue damage and, thus, some markers of renal injury may become relevant.

Inflammation, a hallmark of CKD, is triggered by harmful stimuli, able to activate polymorphonuclear cells and monocytes, which produce several inflammatory cytokines, reactive oxygen metabolites and proteases that can amplify the inflammatory response to a systemic level, by inducing the activation of other inflammatory cells and the production of other cytokines and of several acute-phase proteins. It seems that the persistent inflammation in CKD triggers self-enhancement of the inflammatory cascade and exacerbates wasting and vascular

calcification, amplifying the risk for poor outcome [26]. Actually, inflammation is a morbidity and mortality risk factor for CKD patients. In ESRD patients on HD treatment, the chronic inflammatory state is especially enhanced, as well as vascular calcification, endothelial dysfunction and wasting [27, 28]. Thus, several biomarkers of inflammation have been largely studied as predictive markers of CVD risk and mortality in CKD patients.

The inflammatory biomarkers, C-reactive protein (CRP), interleukin (IL)-6 and tumor necrosis factor (TNF)-α, have been reported to be enhanced in CKD [2, 29]. According to Chronic Renal Insufficiency Cohort (CRIC) study, the inflammatory biomarkers IL-1β, IL-1 receptor antagonist, IL-6, TNF-α, CRP and fibrinogen, are correlated negatively with markers of kidney function, and positively with albuminuria [30]. A cytokine and a T cell imbalance have been also reported in ESRD [31]. CRP measurement was reported as a good predictor of mortality in HD patients [32], while IL-6 was considered a predictor of all-cause and CVD mortality [33, 34]. In a recent study by our team we found that CRP was an independent risk factor for mortality in HD patients [3].

There are other factors that may contribute to the persistence of inflammation in CKD patients, besides the pro-inflammatory factors released along the inflammatory response. The impairment in immune response, involving neutrophils and T cells, favors the risk of infection [35]. In HD patients, infections, such as catheter-related bloodstream infections and access site infections, as well as thrombotic events, are common and enhance inflammation [36]. An increase in pro-inflammatory cytokines alongside with a reduction in their clearance also favors the pro-inflammatory state. Inadequate antioxidant defenses to face the enhanced production of reactive oxygen species (ROS) may favor the inflammatory milieu. Retention of uremic solutes, such as guanidines, interferes with monocyte/macrophage inflammatory activity, which may favor CVD and infection [37]. Obesity increases the risk for kidney disease in the general population [38] and is associated with an altered production of adipokines and a low-grade inflammatory state. For instance, hyperleptinemia has been associated with several CVD risk factors, namely, inflammation, insulin resistance, protein energy wasting and with progression of CKD [39]. Adiponectin, an anti-inflammatory adipokine that is usually reduced in obesity, is increased in CKD patients, probably due to the development of adiponectin resistance, and has been associated with increased mortality risk [40]. In HD patients the overproduction of pro-inflammatory cytokines, the enhancement in phagocyte oxidative burst, activation of NADPH oxidase and the removal of antioxidants by the dialysis procedure [41], produce an additional inflammatory stimuli.

Malnutrition and protein-energy wasting, common in CKD, may also contribute to the inflammatory condition of CKD patients [42]. Mineral and bone disorders, comorbidities associated with CKD, are also linked to the inflammatory process [42].

The close relationship between inflammation and anemia, a common complication of CKD, is well known. Anemia mainly results from a reduced production of erythropoietin (EPO) by the failing kidneys. The increase of the inflammatory cytokine IL-6 in CKD patients leads to an increase in the production of hepcidin that is able to induce the development of a functional iron deficiency. Hepcidin inhibits iron absorption by the enterocytes, and the mobilization of iron stores, from the macrophages of the reticuloendothelial system,

compromising iron availability for erythropoiesis. The increase of hepcidin often leads to a functional iron deficiency in CKD patients. Iron deficiency, either absolute or functional, can contribute to the development or worsening of anemia in CKD patients [3, 43]. Inflammation is enhanced in patients who develop resistance to recombinant human EPO (rhEPO) therapy; however, the mechanisms responsible for the development of the hyporesponse to rhEPO are not fully understood [5, 43].

Inflammation is also common to other inflammatory conditions, such as aging, obesity, diabetes *mellitus* and CVD. Thus, the coexistence of these diseases with CKD may further enhance inflammation, contributing and/or aggravating the inflammatory-associated complications, namely the risk for CV events [44]. Indeed, several pro-inflammatory cytokines that are enhanced in CKD present proatherogenic properties, such as up-regulation of adhesion molecules, enhancement of endothelial dysfunction, promotion of vascular calcification and insulin resistance, and oxidative stress generation [31].

3. Inflammation and DNA damage

More recently, inflammation and inflammatory conditions, including CKD, have been associated to DNA damage. The positive correlation between the levels of DNA damage and the mortality risk in CKD patients suggests that genomic damage can be valuable for prognosis in these patients [8].

The chronic inflammatory state in CKD patients favor genomic damage, which may be induced by inflammatory products and mediators, as well as by external environmental factors, as those associated to the HD procedure [45]. Unrepaired or incorrectly repaired nuclear or mitochondrial DNA damage leads to cell cycle arrest and apoptosis or to mutations. Mutations include intra- or interstrand cross-links, cross-links between DNA bases and proteins, single-strand breaks (SSB), double-strand breaks (DSB) and oxidized DNA bases. DNA repair capacity is essential to correct DNA damage, reduce the genomic damage and, therefore, to reduce cancer risk that appears to be higher in CKD [4].

The genomic damage can be detected by sensitive biomarkers, like unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), mitotic index, telomere length, mitochondrial DNA, micronucleus (MN) assay, comet assay fluorescence *in situ* hybridization (FISH) with DNA or with protein (Immuno-FISH), comparative genomic hybridization (CGH); array-comparative genomic hybridization (array-CGH), spectral karyotyping (SKY), G-banding and flow cytometry [4, 8, 46–48]. These approaches can be used for the identification of genomic lesions, susceptibility to environmental genotoxins and inadequate DNA repair in CKD and HD patients [46].

3.1. Comet assay

The comet assay or single cell gel electrophoresis (SCGE), introduced in 1984, is a sensitive and simple technique for detecting DNA damage at the level of a single cell, under neutral or

alkaline conditions; this test can be complemented with the use of repair enzymes. This assay is useful for measuring SSB, DSB and alkaline labile sites (ALS) in cells and is dependent on the ability of breaks to relax DNA supercoiling linked to the nuclear matrix [49–51]. Concisely, the comet assay requires a suspension of cells embedded in low melting agarose, cellular lyses (to remove plasmatic membranes, cytosol, nucleoplasm and proteins), DNA denaturation (release of histones from DNA), and electrophoresis at neutral or alkaline conditions, where DNA moves to the anode, in a way that is dependent on the number of lesions in the nucleoid, forming a comet. The neutral method (pH = 8.4) only detects DSB, while the alkaline method (pH > 13), with higher sensitivity, identifies both SSB and DSB [51]. For this procedure, it is important to optimize agarose concentration (0.6–0.8%), alkaline unwinding time (40 minutes) and electrophoresis conditions (time, voltage and current, usually 1.15 V/cm), to achieve reliable data on the degree of DNA damage [52]. After electrophoresis, samples are neutralized, stained with a DNA-binding fluorescence dye and analyzed by fluorescence microscopy [49–51, 53, 54]. The comet is composed by a head that contains the undamaged DNA of the nucleus, and by a comet tail, which includes SSB and DSB [49, 50]. The number of DNA breaks is shown by the intensity and length of the tail to the head of the comet [49]. The percentage of DNA in the tail (%T), the tail length and tail moment, provided by an adequate software, measures the DNA damage. The tail moment represents the product of %T and tail length [55, 56].

The scoring systems for the comet assay can use a computer-based image system (semi-automated or automated) coupled to a microscope, and the results are expressed in arbitrary units (AU). Using this visual scoring system, a total of 100 comets per 2 replicate gels are observed, and each comet is assigned to 1 of 5 classes, according to the tail and head intensity. In class 0, there is no DNA in the tail (undamaged DNA); and from class 1 to class 4 (severe damage), the increase of DNA in the tail is proportional to DNA damage. The average extension of DNA migration is calculated by assigning numerical values to each migration class. The comet scoring into classes should be randomly performed in the gel, avoiding edges and areas/cells close to bubbles or artifacts of the gel; ideally, the same operator should perform all scorings. For each sample, the score is calculated applying the following formula: (percentage of cells in class 0 × 0) + (percentage of cells in class 1 × 1) + (percentage of cells in class 2 × 2) + (percentage of cells in class 3 × 3) + (percentage of cells in class 4 × 4) [57, 58]. Afterwards, DNA damage is calculated in arbitrary units (AU) using the formula:

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$$AU = \frac{0 \times N0 + 1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4 \times 100}{\text{number of analyzed comets}} \quad (1)$$

where N0, N1, N2, N3 and N4 are the numbers of comets in classes 0, 1, 2, 3 and 4, respectively. The values of DNA damage reported in AU may be transformed into estimated percentage of DNA in the tail (E%T), using: E%T = (AU/5) + 10, that converts the visual score to a pseudo-percentage score, ps (ps = vs/5 + 10) in a scale range limited to 10–90% [59]; or the conversion curve E%T = (AU − 25.87)/4.46 [60].

More recently, several methodological modifications of the comet assay were developed to detect and quantify DNA damage. The OpenComet, an automated software tool, allows the quantitative measurement of SSB, DSB, ALS and DNA crosslinks with high accuracy and reproducibility, with the advantage of a shorter analysis time [61]. The CometQ is an innovative,

fully automated tool to analyze the images of comet assay with high accuracy, sensitivity and good predictive positive value [62]. The high throughput comet (HT-COMET) assay provides accuracy, efficiency and gives DNA damage profile that allows the determination of the proportion of highly damaged cells [63]. The Comet-FISH measures the percentage of DNA lesions or DNA modifications in the comet tail, which can be enzymatically or chemically converted into strand breaks, providing a way to study the molecular mechanisms of different repair pathways and the screening of drugs, as potential specific inhibitors for repair pathways [64]. Comparing with the MN assay, the comet assay allows the study of non-proliferating cells and does not need to use cell cultures [4]. However, the assay of MN, also known as Howell-Jolly bodies, is recognized as robust, sensitive, fast and reliable method, which analyses cytogenetic damage, namely, chromosomal breaks (clastogenesis), disruptions of mitotic apparatus with chromosomal losses (aneugenesis) and amplifications [51, 53].

3.2. Cell-free DNA

Human plasma contains cell free nucleic acids, including genomic DNA, mitochondrial DNA, mRNAs and miRNAS, all with different functions [65]. DNA is released following cell damage and is raised in several clinical conditions, such as diabetes, trauma, cancer, systemic lupus erythematosus, age-associated inflammation and inflammation-associated diseases [65, 66].

In diabetes *mellitus*, one of the most common causes of CKD, cfDNA levels were reported to be increased, both in patients with and without microvascular complications, though higher in those with microvascular disturbances [67]. It was hypothesized that in diabetes, the reactive oxygen and nitrogen species cause DNA strand-breakage, which may activate the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) [68, 69]. The activation of PARP induces depletion of DNA, reducing glycolysis, electron transport and ATP formation; moreover, it inhibits the synthesis of glyceraldehyde 3-phophate by poly-ADP-ribosylation dehydrogenase. All these mechanisms seem to lead to acute endothelial dysfunction, favoring the development of diabetic complications [67].

As referred, inflammation is a hallmark of CKD and is particularly enhanced in ESRD patients under HD. The underlying inflammatory process might contribute to increase DNA damage [66]. In ESRD patients on HD, the cellular necrosis and apoptosis occurring along the HD process [70], the enhanced production of ROS and toxins, such as advanced glycation end products derived from oxidative peroxidation [71, 72], may contribute to a higher rise in cfDNA levels. Modifications in DNA repair mechanisms may also favor the increase of DNA damage [8]. Epigenetic variations, including DNA methylation patterns, histone modifications, chromatin remodeling, microRNAs and long non-coding RNAs, can change the flow of gene expression, acting as genotoxic modifiers by promoting DNA damage and chromosome abnormalities [51].

The traditional method for DNA quantification is the ultraviolet absorbance spectroscopy assay, which is not applicable to biological samples. In this case, after DNA extraction from the biological fluid, cfDNA can be quantified, using specific dyes, by colorimetry or emission fluorometry; however, these methods are complex and expensive. Goldshtein et al. [73] developed a simple, inexpensive and accurate test for cfDNA evaluation that does not require prior

processing of samples. Briefly, SYBR® Gold stain is diluted in dimethyl sulfoxide (1:1000 dilution) and phosphate buffer (1:8 dilution); the biological fluid (serum, whole blood, urine or supernatant of cell cultures) is mixed with SYBR® Gold solution (final stain dilution: 1:10,000) and cfDNA fluorescence is measured with a fluorimeter (emission wavelength 535 nm, excitation wavelength of 488 nm). Czeiger et al. applied this method to a study using an animal model and patients with colorectal cancer; and found that mice inoculated with patient's cancer cells, presented a positive correlation between cfDNA and tumor size [74]; comparing cfDNA levels between controls and preoperative patients, cfDNA levels were higher in patients; 1 year after, the levels of cfDNA were higher in patients who remained with the disease or died, as compared with those without disease; in accordance, the authors proposed that in colorectal cancer patients the levels of cfDNA had a prognostic value, for death and for the outcome of the disease [74].

4. DNA damage in ESRD patients

Our team has been interested in studying DNA damage and its correlation with the enhanced inflammatory state observed in different inflammatory conditions, as in ESRD under HD treatment and in psoriasis *vulgaris*; in both these clinical conditions we found that cfDNA levels were increased and correlated with inflammatory markers, as IL-6 and CRP in ESRD [66]; and, in psoriasis, cfDNA levels were correlated positively with IL-6, suggesting a linkage with psoriasis severity [75].

In a more recent work, we analyzed the degree of genomic damage in ESRD patients under HD therapy for more than 1 year, using two different approaches, the alkaline *in vitro* comet assay and the cfDNA quantification (according to Goldshtein et al. method [73]), in order to evaluate DNA damage within the cell and the circulating free DNA, respectively. We studied 39 ESRD patients (24 males and 15 females with a median age of 68, [58–77] interquartile ranges) that were under HD therapeutic, 2–3 times per week, 3–5 hours each HD session, for a median time of 67, [40–94] months; high-flux polysulfone FX-class dialyser of Frenesius (Bad Hamburg, Germany) was used for the HD procedure. The main causes of renal failure were diabetic nephropathy (n = 12), hypertensive nephrosclerosis (n = 11), pyelonephritis (n = 5), IgA nephropathy (n = 4), polycystic kidney disease (n = 3), other diseases (n = 2) and of uncertain etiology (n = 2). Besides rhEPO therapy, patients were under iron and folate prophylactic therapies, in accordance to the recommendations of "KDIGO Clinical Practice Guideline for Anemia in Chronic Kidney Disease" [76], to avoid nutrient erythropoietic deficiencies. A group of 15 healthy volunteers, 2 males and 13 females, with normal hematological and biochemical values, without history of renal or inflammatory diseases, was also studied. This control group was matched as far as possible for age, once the age of HD patients is usually high. ESRD patients and controls were matched for body mass index, but not for gender (**Table 2**).

We found that ESRD patients presented significantly lower values of erythrocytes, hemoglobin concentration and hematocrit; the erythrocytes were less hemoglobinized, as showed by the significantly lower value of mean cell hemoglobin concentration; however, iron stores were increased, as ferritin was significantly increased (about sixfold the control

	Controls (n = 15)	ERSD patients (n = 39)	P-value
Sociodemographic data			
Age (years)	52 [40–55]	68 [58–77]	0.001
Gender [(M/F); n (%)]	2 (13%)/13 (87%)	24 (62%)/15 (38%)	0.002
BMI (kg/m^2)	22.9 [20.6–27.2]	25.2 [21.5–27.8]	0.329
Hematologic data			
RBC (×10^{12}/l)	4.60 [4.20–5.00]	3.70 [3.50–3.90]	<0.001
Ht (%)	40.3 ± 4.7	35.8 ± 4.1	0.001
Hb (g/dl)	13.6 ± 1.5	11.8 ± 1.5	<0.001
MCV (fl)	89.0 [84.0–94.0]	96.9 [95.2–99.2]	<0.001
MCH (pg)	30.7 [28.1–31.6]	31.9 [30.8–32.6]	0.006
MCHC (g/dl)	33.7 ± 1.1	32.8 ± 1.1	0.008
WBC (×10^9/l)	7.3 [5.4–8.1]	5.8 [5.1–7.7]	0.164
Biochemical data			
Iron (μg/dl)	69.5 [64.5–110.8]	65.0 [56.0–87.0]	0.299
Ferritin (μg/dl)	68 [15–137]	461 [351–680]	<0.001
Transferrin (mg/dl)	307 [238–338]	173 [158–194]	<0.001
Transferrin saturation (%)	20.5 [15.9–26.7]	27.8 [22.2–42.5]	0.008
CRP (mg/l)	0.7 [0.6–0.4]	2.9 [1.7–12.5]	0.017
Cell-free DNA (ng/ml)	116 [90–267]	371 [217–563]	0.002*

BMI, body mass index; CRP, C-reactive protein; F, female; Ht, hematocrit; Hb, hemoglobin; M, male; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RBC, red blood cell; [illegible] 0.05 was accepted as statistically significant.

Results are presented as mean ± standard deviation or as median [interquartile range]; differences between groups were tested using chi-squared test and Fisher's exact test for categorical variables; for continuous variables, the unpaired Student's t-test or the Mann-Whitney U test were used, according to the distribution of the variable.

*Loss of significance after statistical adjustment for age (analysis of covariance (ANCOVA)).

Table 2. Sociodemographic data, hematologic, biochemical, and cell-free DNA values in end-stage renal disease (ERSD) patients and controls.

value). These findings suggest a functional iron deficiency that seems to be linked to the high inflammatory state observed in ERSD patients, with significantly higher CRP values (**Table 2**). Considering that inflammation regulates iron absorption and iron availability for hemoglobin synthesis, the enhanced inflammatory state in ESRD patients contributes to worsening of anemia and to the reduction in erythrocyte hemoglobinization.

We used the comet assay to evaluate DNA structural damage in blood cells from controls and ESRD patients on HD. The distribution of comets was obtained by visual scoring into five classes (**Figure 1**), based on the length of migration and/or in the relative proportion of DNA in the head and in the tail [57, 58].

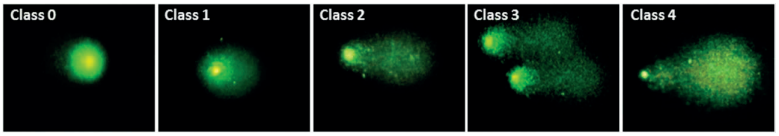

Figure 1. Comet images of lymphocytes from end-stage renal disease (ERSD) patients showing different migration patterns, according to the levels of DNA damage, from class 0 (undamaged) to class 4 (severe damage).

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DNA damage presented as AU, for controls and patients, are displayed in **Figure 2**. We found that DNA damage (AU) was significantly higher in ESRD patients (71 [36–127]), when compared to controls (34 [0–61]). A significant increase was also observed for %T in ESRD patients, when compared to controls. We found a %T of 24.2 [17.2–35.4] and 16.8 [10.0–22.2] (expressed in pseudo percentage), and 10.12 [2.27–22.71] and 1.82 [−5.80–7.88] (using the conversion curve) for patients and controls, respectively (**Figure 3**). The conversion curve provides a better fitting between %T and AU [55, 60] and showed negative values of %T for AU below 26, indicating that %T was zero; above 400 AU, the %T was 84%, in accordance with others [55, 60]. Our data is in accordance with other studies reporting that the levels of DNA breaks and oxidative DNA lesions, measured by the comet assay, are higher in dialysis patients then in controls [77].

We also found that %T was negatively correlated (Spearman's rank correlation) with CRP ($r = -0.368$; $P = 0.021$) and ferritin ($r = -0.404$; $P = 0.011$), in ESRD patients; no significant correlations were found between DNA lesions and the rhEPO dose used to treat anemia ($r = 0.171$; $P = 0.306$), or the time of HD treatment ($r = -0.186$; $P = 0.256$). In a cross-sectional study, the oxidative DNA lesions found in dialysis patients were inversely correlated with the duration of the dialysis sessions [77, 78].

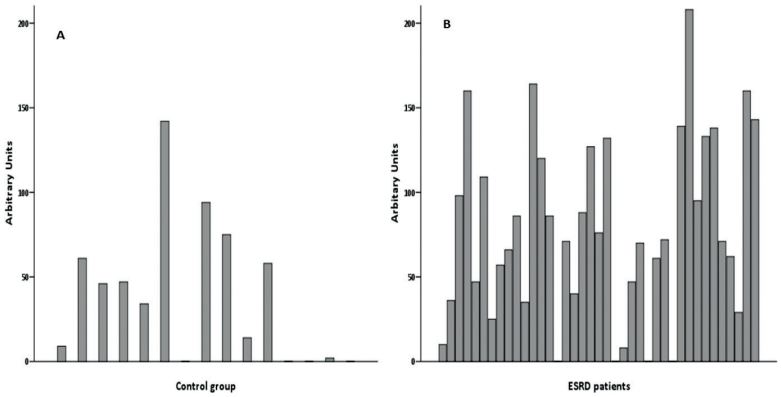

Figure 2. DNA damage, presented in arbitrary units, for each of the 15 healthy controls (A) and for the 39 ESRD patients (B).

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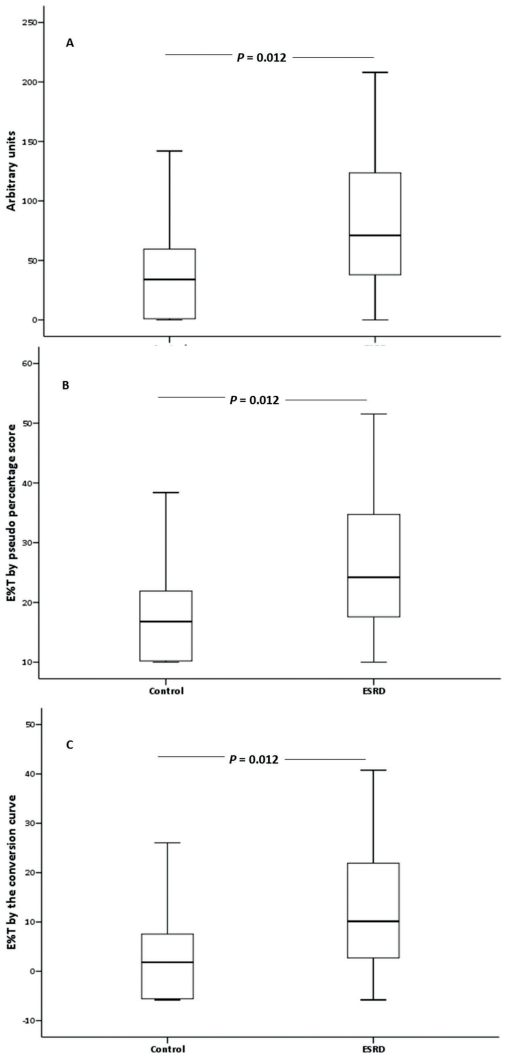

02 **Figure 3.** (A) Mean values in arbitrary units and the estimated percentage of DNA in the tail (E%T) calculated using two equations: (B) the pseudo percentage score (ps = vs/5 + 10) and (C) the conversion curve = (AU – 25.87)/4.46, in controls and end-stage renal disease (ESRD) patients (differences between groups were tested using Mann-Whitney U test).

We did not find significant differences in DNA damage (comet tail length or tail intensity) for diabetics and nondiabetic ESRD patients, as reported by Ersson et al. [77]; however, our findings are in accordance with Mamur et al., reporting no difference in comet tail length or tail intensity between diabetic and non-diabetic ESRD patients on HD [78].

Our data suggest that long-term dialysis treatment or diabetes *mellitus* do not affect DNA damage, however there are still few studies and controversial data. Ersson et al. reported lower levels of DNA damage in salivary gland tissues of ESRD patients, as compared to controls, suggesting that ESRD might affect DNA in different ways, in peripheral tissues and in blood mononuclear cells [77].

Concerning cell-free DNA, we found that ESRD patients had a significantly higher value, compared to control; however, after statistical adjustment for age, the significance was lost (**Table 2**). Cell-free DNA correlated (Spearman's rank correlation) significantly and positively with age in both groups ($r = 0.342$, $P = 0.033$; $r = 0.589$, $P = 0.021$; in patients and controls, respectively), and with CRP in ESRD patients ($r = 0.483$; $P = 0.002$). Our results are in accordance with others reporting increased levels of cfDNA in hemodialyzed patients [79].

To study the predictive risk of mortality associated with DNA damage, we recorded the number of deaths that occurred along 1 year after the analytical study of the 39 ESRD patients; 9 out of the 39 ESRD patients died. We compared the analytical data from ESRD patients who were alive and from the patients who died in the 1 year follow-up period (the Mann-Whitney U test was used). The latter patients presented significantly higher ($P = 0.006$) cfDNA values (713 [415–809] ng/ml) than those who were still alive (337 [192–484] ng/ml). A trend towards ($P = 0.149$) higher CRP levels (7.5 [1.6–45.7] mg/l) in those who died, compared to those who were still alive (2.7 [1.7–9.3] mg/l), was also found.

The differences in DNA damage, observed between controls and ERSD patients, could be higher, if we were able to gather a gender matched population. It is known that DNA lesions are higher in women, both in healthy [43, 80–82] and in pathological conditions [83].

Divergent results have been reported for the levels of DNA damage and the time of dialysis treatment. Some studies showed a reduction of DNA damage on long-term maintenance HD [84, 85], while others showed an increase [8, 86, 87]. Recently, it was reported that online hemodiafiltration (OL-HDF) reduced the levels of DNA damage in these patients, as this approach provides a reduction of inflammation and oxidative stress [10]. In fact, a reduction of binucleated cells with micronuclei in patients that changed from low-flux HD to post-dilution OL-HDF, as well as an increase in plasma antioxidant capacity, were shown [88]. Both single high-flux HD and OL-HDF remove circulating mitochondrial DNA, a pro-inflammatory agent, which has been correlated with the chronic inflammatory grade of hemodialyzed patients [89]. Moreover, OL-HDF procedure has been associated with lower levels of the inflammatory markers, IL-6 and CRP, and with an improvement on endothelial (dys)function, in ESRD patients [90, 91]. Aberrant DNA hypermethylation has been also observed in dialysis patients and associated with the inflammatory state and with the

dialysis technique; patients under OL-HDF showed lower DNA methylation patterns than patients under HD, although higher than controls, suggesting a reduction in DNA hypermethylation, with decreasing inflammation [92].

Dietary supplementation with folic acid [87, 93], vitamins A, B and B12 [93], zinc [94] and selenium [87] may also contribute to reduce/avoid genomic damage, once nutritional supplementation has antioxidant effects, prevents cancer, increases DNA repair capacity, and improves CV and all-cause mortality rates [87].

The inverse correlation that we observed between %T and CRP in ESRD patients, suggests that as CRP (inflammation) levels increase, the damage in DNA also increases; however, it seems that for lower CRP values the damaged DNA is still within the cell, while at higher CRP values the increasing damaged DNA is released into plasma.

The increase of cfDNA in ESRD patients was also reported by others [5, 65, 66, 70, 79]. The slightly lower cfDNA values found in our study, compared with those found by others in HD patients [5], may be related with time of sample collection, as the levels of cfDNA increase during and after HD, returning to pre-HD levels half an hour post-HD [95].

We should notice that our study has some limitations, namely, the small sample size, the lack of age and gender matched controls. Thus, further studies in larger populations are needed to strengthen the value of cfDNA as a biomarker of inflammation and poor outcome in ESRD patients. A recent study showed that circulating free DNA, by favoring calcium phosphate precipitation and crystallization, may be involved in arterial calcification [96], a common feature in ESRD patients under HD. Thus, cfDNA, appears to be a biomarker for CVD risk, and a direct contributor for CV events, the main cause of death in ERSD patients.

5. Conclusions remarks

ESRD is characterized by a low-grade chronic inflammatory state, which favors the development of comorbidities. Genetic damage has been reported in ESRD patients, especially in those under HD. The higher degree of genomic damage in ESRD patients might be a consequence of inflammation and aging, and may contribute to increase the risk for cancer and cardiovascular mortality. Several associations with DNA damage (evaluated by cfDNA and comet assay) have been reported and support this hypothesis; however, data is limited and controversial (**Table 3**).

Our studies showed that cell damaged DNA is increased in ESRD patients, and suggest that at lower CRP values the damaged DNA remains within the cell, while at higher CRP values damaged DNA is released into plasma and may contribute to further enhance inflammation in ESRD patients and increase mortality risk. Actually, we found that ESRD patients who died within the one year follow-up period of the study, presented higher circulating damaged DNA

Comet assay		
Positive association	**Negative association**	**No association**
Male gender [9]	CRP*	Gender [78]
Diabetes [9]	Ferritin*	Diabetes [78]*
Mortality [8, 97]	Dialysis sessions duration [77]	Duration of HD [78]*
Frequency of micronuclei [98]		Ferritin [78]
BMI > 25 kg/m^2 [78]		Age [78]
Intact PTH > 300 pg/ml [78]		Hb [78]
Leptin [99]		Hypertension [78]
Treatment modality [9]		rhEPO dose*

cfDNA	
Positive association	**No association**
Age*	TNF-α [70]
CRP [66]*	IL-10 [70]
IL-6 [66, 70]	Dialysis duration [100]
All-cause mortality (post-dialysis) [5]	WBC count (before HD) [100]
Last 3-month mean: SBP, WBC, serum albumin, Cr, normalized protein catabolic rate [101]	Length of the HD session [95]
In HD diabetic patients: SBP, Hb A1c, and serum albumin [101]	

BMI, body mass index; Cr, creatinine; CRP, C-reactive protein; Hb, hemoglobin; IL, interleukin; PTH, parathyroid hormone; rhEPO, recombinant human erythropoietin; SBP, systolic blood pressure; TNF, tumor necrosis factor; WBC, white blood cell.

*According to our data.

Table 3. Associations reported for comet assay and cell-free (cf) DNA on hemodialysis (HD) patients [96–101].

and inflammation. Moreover, our data suggest that the comet assay is more sensitive for low grade inflammatory conditions, while cfDNA appears as a good biomarker for more severe inflammatory conditions, as well as a biomarker for the outcome of ESRD patients. In summary, the genomic damage in ESRD patients seems to result, at least in part, from inflammation and aging, and may contribute to increase the risk for cancer and CV mortality.

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Author details

Susana Coimbra[1,2], Alice Santos-Silva[3]*, Elísio Costa[3] and Elsa Bronze-da-Rocha[3]

*Address all correspondence to: assilva@ff.up.pt

1 CESPU, Institute of Research and Advanced Training in Health Sciences and Technologies (IINFACTS), Gandra-PRD, Portugal

2 UCIBIO\REQUIMTE, Porto, Portugal

3 UCIBIO\REQUIMTE, Department of Biological Sciences, Laboratory of Biochemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal

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